Article

Nonprotonophoric Electrogenic Cl⁻ Transport Mediated by Valinomycin-like Carriers



Synthetic anion transporters can facilitate H⁺ transport via deprotonation, or OH⁻ transport via hydrogen bonding to OH⁻, thus allowing dissipation of transmembrane pH gradients, an undesired side-effect for biomedical applications as Cl⁻ ionophores. To address this limitation, Gale and colleagues have developed two anionophores that show high Cl⁻ > H⁺/OH⁻ selectivity. Preliminary cellular studies support the biological relevance of the selectivity.

Xin Wu, Luke W. Judd, Ethan N.W. Howe, ..., Yun-Bao Jiang, Anthony P. Davis, Philip A. Gale

philip.gale@soton.ac.uk (P.A.G.) anthony.davis@bristol.ac.uk (A.P.D.)

HIGHLIGHTS

Anionophores can function via electrogenic or electroneutral mechanisms

Most synthetic anionophores also function as protonophores

Proton and hydroxide transport are two mechanisms for protonophoric activity

Anion encapsulation confers selectivity for chloride over protons/hydroxide

ACCESSION NUMBERS CCDC: 1431251

Wu et al., Chem 1, 127–146 July 7, 2016 © 2016 The Author(s). Published by Elsevier Inc. http://dx.doi.org/10.1016/j.chempr.2016.04.002





Article

Nonprotonophoric Electrogenic Cl⁻ Transport Mediated by Valinomycin-like Carriers

Xin Wu,¹ Luke W. Judd,² Ethan N.W. Howe,¹ Anne M. Withecombe,¹ Vanessa Soto-Cerrato,³ Hongyu Li,⁴ Nathalie Busschaert,^{1,6} Hennie Valkenier,^{2,7} Ricardo Pérez-Tomás,³ David N. Sheppard,⁴ Yun-Bao Jiang,⁵ Anthony P. Davis,^{2,*} and Philip A. Gale^{1,8,*}

SUMMARY

Synthetic transmembrane anion transporters (anionophores) have potential as tools for biomedical research and as therapeutic agents for diseases associated with anion-channel dysfunction. However, the possibility of H⁺ or OH⁻ transport by anionophores has received little attention, and an anionophore selective for Cl⁻ over H⁺/OH⁻ is currently unavailable. Here, we show that depending on anionophore acidity, many anionophores facilitate electrogenic H⁺ or OH⁻ transport, potentially leading to toxicity. Nevertheless, using several liposome-membrane-based assays, we identified two newly developed small molecules that promote electrogenic Cl⁻ transport without effectively dissipating the transmembrane pH gradient, essentially mimicking the electrogenic cationophore valinomycin. The Cl⁻ > H⁺/OH⁻ selectivity of anionophores showed a consistent positive correlation with the degree of Cl⁻ encapsulation and a negative correlation with the acidity of hydrogen-bond donors. Our study demonstrates that a valinomycin equivalent for Cl⁻-selective transport is achievable.

INTRODUCTION

Facilitation of transmembrane ion transport by small-molecule carriers that function by reversible binding of the transported ionic species is an important supramolecular process.^{1,2} The most notable example of a small-molecule carrier is the naturally occurring K⁺ carrier valinomycin (Figure 1), which has found many applications in the study of biological systems.³ Valinomycin facilitates transmembrane K⁺ transport by interfacial binding of K⁺, translocation of the cationic complex through the lipid membrane, release of K⁺ at the other interface of the membrane, and eventually back transport of the uncomplexed valinomycin to complete the cycle (Figure 2B, left).^{4,5} The action of valinomycin is electrogenic because there is a net transfer of charge across the membrane. Ionophores, such as valinomycin, that transfer charge across the membrane are termed "electrogenic" (i.e., they modify a membrane potential) or "electrophoretic" (i.e., they transport ions driven by a membrane potential). The more common term "electrogenic" is used throughout this article. By contrast, the action of some other cationophores is purely electroneutral (i.e., there is no movement of net charge) because a metal ion (M⁺) is exchanged for H⁺ (Figure 2A, left). One example is the carboxylate-containing cationophore monensin,⁶ which carries a monovalent cation through the membrane as an overall neutral ion-pair complex (Figure 1) with no transmembrane movement of charged species.³ Valinomycin and monensin thus have fundamentally different iontransport mechanisms controlled via membrane potential (valinomycin) or pH gradient (monensin).³

The Bigger Picture

The movement of ions and molecules through biological membranes is regulated by embedded protein channels, carriers, and pumps. The dysfunction of ion channels is the underlying cause of many diseases, notably cystic fibrosis, in which passive chloride ion transport is impaired. A number of groups are making small molecules that can carry chloride through lipid-bilayer membranes and so potentially replace the function of faulty channels, thereby ameliorating the symptoms of these types of diseases. However, many of these transporters exhibit a side effect of pH-gradient disruption, which can lead to cell death. Herein, we show the first examples of transporters with a high selectivity for chloride over proton and hydroxide, which represents a paradigm shift for transporter design. This is a significant step toward real biomedical application of anion transporters in the battle against cystic fibrosis and other diseases caused by faulty ion channels.



Although the transport properties of naturally occurring cationophores, many of which possess antibiotic properties, are well understood, natural or synthetic transmembrane anion transporters (anionophores) have only recently attracted significant attention.^{7,8} Anionophores are useful in complementing valinomycin and other cationophores in biomedical research^{9,10} and might be used to replace defective anion channels in treatments for genetic diseases such as cystic fibrosis^{11,12} or to induce cancer cell apoptosis by facilitating NaCl influx.¹³ The best known anionophore, the natural product prodigiosin (Figure 1), facilitates H⁺/Cl⁻ symport^{14,15} (cotransport; Figure 2A, right) and Cl⁻/NO₃⁻ antiport (exchange),¹⁶ but its non-electrogenic nature, reported by Sato et al.,¹⁷ is sometimes overlooked. A wide spectrum of synthetic anionophores that function by hydrogen bonding,^{7,8} halogen bonding,¹⁸ or metal coordination¹⁹ to anions have been developed. So far, research efforts have mainly focused on their efficacy in facilitating anion exchange, and little attention has been paid to anion-transport mechanisms, especially their ability to disrupt pH or proton gradients.

Transmembrane pH gradients are essential for cellular function.²⁰ It is therefore important to understand the role ionophores play in facilitating proton or hydroxide transport. (H⁺ transport produces the same effect as OH⁻ transport in the reverse direction. In this article, we use the term " H^+/OH^- transport" to refer to the process of H⁺ and/or OH⁻ translocation through lipid bilayers. We also use "/" elsewhere to indicate a coupled process, e.g., "H⁺/Cl⁻ symport.") In this respect, valinomycin functions as a selective electrogenic K⁺ ionophore that does not facilitate H⁺/OH⁻ transport.²¹ By contrast, currently no anionophore has been identified with strong $Cl^- > H^+/OH^-$ selectivity.²² Indeed, prodigiosin,^{14,15} and some of the most powerful synthetic anionophores,²³⁻²⁷ have been shown to uncouple vacuolar type H⁺-ATPase (V-ATPase) and neutralize acidic cellular organelles (such as the Golgi apparatus, lysosomes, and endosomes) by facilitating H^+/Cl^- symport or Cl⁻/OH⁻ antiport through organelle membranes. Monensin is also known to exhibit this neutralization effect by promoting Na⁺/H⁺ antiport through organelle membranes.⁶ Another possible yet unexamined action of synthetic anionophores is electrogenic H⁺ transport. Compounds known as "protonophores," such as carbonyl cyanide phenylhydrazones,²⁸ facilitate electrogenic H⁺ transport (Figure 2C, left), enabling them to uncouple oxidative phosphorylation by dissipating the proton gradient pumped by the electron transport chain.²⁹

Although prodigiosin and other functionally similar compounds are promising anticancer agents because of their ability to disrupt transmembrane pH gradients, electrogenic Cl⁻ carriers that do not facilitate H^+/OH^- transport ("valinomycin-like" anionophores) are required for other applications. These include using anionophores for physiological research where H^+/OH^- transport would complicate data interpretation. Examples include the identification of electrogenic H⁺-coupled metal-ion transport, such as H^+/K^+ symport (here the aninophore would dissipate the accumulation of a membrane potential like the use of valinomycin in the study of Cl⁻/H⁺ antiport by ClC transporters³⁰); and potential replacement of defective anion channels in genetic diseases where H^+/OH^- transport would lead to toxicity (e.g., Best disease, Startle disease, Bartter syndrome, and most notably, cystic fibrosis).³¹

As a process functionally equivalent to H⁺ transport,³² OH⁻ transport, at first glance, seems an unlikely process for synthetic anionophores to facilitate, considering the high energy penalty required to dehydrate OH⁻ ($\Delta G_{hydration}(OH^-) = -430 \text{ kJ mol}^{-1}$ compared with $\Delta G_{hydration}(CI^-) = -340 \text{ kJ mol}^{-1}$).³³ However, OH⁻

¹Chemistry, University of Southampton, Southampton SO17 1BJ, UK

²School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, UK

³Cancer Cell Biology Research Group, Department of Pathology and Experimental Therapeutics, Faculty of Medicine, Universitat de Barcelona, 08907 Barcelona, Spain

⁴School of Physiology, Pharmacology and Neuroscience, University of Bristol, Biomedical Sciences Building, University Walk, Bristol BS8 1TD, UK

⁵Department of Chemistry, College of Chemistry and Chemical Engineering, MOE Key Laboratory of Spectrochemical Analysis and Instrumentation, Xiamen University, Xiamen 361005, China

⁶Present address: Department of Chemistry, New York University, Silver Center for Arts and Science, 100 Washington Square East, 10th Floor, New York, NY 10003, USA

⁷Present address: Université Libre de Bruxelles, CP165/64, Avenue F.D. Roosevelt 50, 1050 Bruxelles, Belgium

⁸Lead Contact

*Correspondence: philip.gale@soton.ac.uk (P.A.G.)

*Correspondence: anthony.davis@bristol.ac.uk (A.P.D.)

http://dx.doi.org/10.1016/j.chempr.2016.04.002



Figure 1. Structures of Anionophores and Other Reagents Used

Novel anionophores are marked with an asterisk. For trihexylamine, prodigiosin, and MnTPP, their Cl $^-$ complexed forms are shown.

CellPress



Figure 2. Schematic Representations of Ion-Transport Mechanisms

(A) Electroneutral M^+/H^+ antiport facilitated by monensin (Mon, left) and electroneutral H^+/Cl^- symport facilitated by prodigiosin (Prod, right).

(B) Electrogenic K⁺ transport facilitated by valinomycin (VIn, left) and electrogenic CI⁻ transport facilitated by an electrogenic anion carrier (right).

(C) Electrogenic H⁺ transport facilitated by a weak acid protonophore (HA, left) and electrogenic OH⁻ transport facilitated by an electrogenic anion carrier (right). Note that these two processes are functionally equivalent.

(D) Equilibria involving hydrogen bonding to OH^- and deprotonation of ureas and thioureas. Hydrogen bonds are represented by red dashed lines.

is an extremely strongly coordinating anion, and previous studies have demonstrated that synthetic anionophores, including halogen-bond-based transporters that do not possess H⁺ binding groups, can dissipate transmembrane pH gradients in which OH⁻ transport coupled with Cl⁻ transport appears to be the only plausible mechanism.¹⁸ In fact, the routine use of the 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) assay, which measures the rate of pH-gradient dissipation, to evaluate

anion-transport activity suggests that designing a Cl^- -selective anionophore that does not facilitate OH^- (or H^+) transport is likely to be challenging.

In this article, we address H⁺/OH⁻ transport facilitated by anionophores. We provide unambiguous evidence that (1) prodigiosin cannot facilitate electrogenic transport of Cl⁻, H⁺, or OH⁻ and (2) many synthetic anionophores can facilitate electrogenic H⁺/OH⁻ transport. We propose that the actual transport process responsible for the observed electrogenic transport of H⁺ or OH⁻ likely depends on the acidity of hydrogen-bond donors in the anionophore. Most importantly, we have developed two "valinomycin-like" electrogenic anionophores that show outstanding Cl⁻ > H⁺/OH⁻ selectivity. One of these compounds was identified as an active anionophore functioning in live cells. Distinct from other active anionophores reported, this selective anionophore did not effectively neutralize lyso-somal pH.

RESULTS AND DISCUSSION

Evidence of Electrogenic H⁺/OH⁻ Transport

Previously, Busschaert et al.³⁴ and Vargas Jentzsch et al.¹⁸ have demonstrated that anionophores dissipate a transmembrane pH gradient in the presence of Cl⁻ in vesicle-based experiments employing the intravesicular pH indicator HPTS (Figure 1).³⁵ Provided that the anionophores do not cause membrane defects or HPTS leakage, these data indicate that anionophores facilitate H⁺/OH⁻ transport (accompanied by Cl⁻ transport as the counterion pathway to maintain electroneutrality). However, an alternative mechanism of H⁺ transport via simple (unassisted) diffusion coupled with Cl⁻ transport facilitated by anionophores has been proposed, ³⁶ and in theory pH-buffer transport is also possible. Assuming H⁺/Cl⁻ symport, such a process might be an obligatorily coupled electroneutral process (Figure 2A, right). In this case, because H⁺ transport cannot be separated from Cl⁻ transport, the anionophore is unable to facilitate electrogenic H⁺ transport (Figure 2C). Alternatively, H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport can be an indirectly coupled process with separate pathways for electrogenic H⁺ (or OH⁻) transport (Figure 2C) and electrogenic Cl⁻ transport (Figure 2B, right). To the best of our knowledge, this possibility has never been examined experimentally in the literature.

To identify the mechanism of H⁺/OH⁻ transport by anionophores, a new HPTS assay (here termed the tetrabutylammonium hydroxide [TBAOH] assay; Figure 3A) was devised for testing purely electrogenic H⁺/OH⁻ transport (in other words, protonophoric activity), in which ionophore-induced dissipation of a pH gradient across large unilamellar vesicle (LUV) membranes (induced by adding 5 mM TBAOH to a vesicle suspension) in a lightly buffered sodium p-gluconate (100 mM) medium was monitored. In this assay, gluconate (Figure 1) transport is negligible because of its large size and hydrophilicity. The presence of TBA⁺, a membrane-permeable cation,³⁷ provides a counterion pathway for electrogenic H⁺/OH⁻ transport (driven by the pH gradient), which would otherwise build up an opposing membrane potential preventing bulk pH change. This leads to overall TBA⁺/OH⁻ symport or TBA⁺/H⁺ antiport in the presence of an electrogenic H⁺/OH⁻ ionophore, dissipating the pH gradient. Under our experimental conditions, the low H⁺ permeability of intact lipid bilayers does not allow for observable pH-gradient dissipation over the timescale of several minutes (see DMSO control in Figure S23).

Results from the TBAOH assay were compared with data from another HPTS assay (termed the *N*-methyl-D-glucamine chloride [NMDG-CI] assay; Figure 3B, left) set

CellPress



${f c}$ KCl efflux - anionophore coupling with Vln

D KCl efflux - anionophore coupling with Mon



Figure 3. LUV-Based Assays Used in This Study

(A) TBAOH assay. Electrogenic H⁺/OH⁻ transport facilitated by the test ionophore couples with simple diffusion of tetrabutylammonium ion (TBA⁺), leading to pH-gradient dissipation (measured by intravesicular pH indicator HPTS) by TBA⁺/H⁺ antiport or TBA⁺/OH⁻ symport (shown in brackets).

(B) NMDG-Cl assay in the absence (left) and presence (right) of proton channel gramicidin D (Gra). In the absence of gramicidin, the test anionophore facilitates H^+/Cl^- symport or $Cl^-/OH^$ antiport (shown in brackets) to dissipate the pH gradient (measured by HPTS). In the presence of gramicidin, which facilitates H^+ transport, the test anionophore is only required to facilitate $Cl^$ transport to dissipate the pH gradient. If H^+/OH^- transport is rate limiting with the test anionophore alone (i.e., if the test anionophore shows $Cl^- > H^+/OH^-$ selectivity), gramicidin will improve the rate-limiting step and thereby accelerate the rate of pH-gradient dissipation. (C) Anionophore-facilitated electrogenic Cl^- transport coupling with valinomycin (VIn) facilitated electrogenic K⁺ transport, leading to net KCl efflux (measured using a Cl^- -selective electrode). (D) Anionophore-facilitated H^+/Cl^- symport (or Cl^-/OH^- antiport shown in brackets) coupling with monensin (Mon) facilitated K⁺/H⁺ antiport, leading to net KCl efflux (measured by a Cl^- -selective electrode). The buffering agent HEPES is omitted for clarity.

up for H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport, in which NMDG-Cl (100 mM) was used in place of sodium gluconate (100 mM), and NMDG (5 mM) was used in place of TBAOH (5 mM) as the base added to create a pH gradient. The conditions used for the NMDG-Cl assay were similar to those of the HPTS assays employed by Vargas Jentzsch et al.,¹⁸ but NMDG-Cl was used instead of NaCl for the sake of another selectivity assay described below. Control experiments were conducted to ensure that the HPTS response represented transport of Cl⁻ and H⁺/OH⁻ and not other processes such as Na⁺/H⁺ antiport, gluconate/OH⁻ antiport, or HPTS leakage from vesicles (Figures S31, S32, and S57–S60). After test ionophores were studied at different concentrations, Hill plot analyses were performed to obtain a Hill coefficient, which indicated the stoichiometry of the unstable³⁸ active species mediating ion transport, and the effective concentration to reach 50% of maximum transport at 200 s (EC₅₀ value, low EC₅₀ values indicate high activity) to quantify ion-transport activity.³⁵

Compound	р <i>К</i> а	EPN ^a (<i>E</i> _h)	TBAOH	l Assay ^b	NMDG-Cl Assay ^c	
			n	EC ₅₀ (mol %)	n	EC ₅₀ (mol %)
1	13.8 ^d	-18.2786	0.9	0.065	1.4	0.046
2	ND ^e	-18.2798	1.9	0.83	2.4	0.61
3	16.1 ^d	-18.2947	1.5	0.26	2.3	0.16
4	8.5 ^d , 8.9 ^f	-18.2723	0.9	0.014	1.2	0.015
5	10.7 ^f	-18.2774	1.1	0.013	1.9	0.26
6	ND ^e	-18.3453	2.9	12	3.2	20
Prodigiosin	ND ^e	ND ^e	_a	>1 ^g	1.4	0.000061
CCCP	6.0 ^h	ND ^d	1.0	0.0054	_ ⁱ	>100 ⁱ

Table 1. Summary of Acidity and Membrane-Transport Data of Ureas/Thioureas 1–6, Prodigiosin, and CCCP

^aElectrostatic potential at the nitrogen nucleus is expressed in atomic units. The structures were optimized using the PM6 semi-empirical method, assuming the *syn* conformer. The EPN values were calculated at the B3LYP/6-311++G(d,p) level of theory with the SMD water-solvation model.

^bA fluorescence assay for electrogenic H⁺/OH⁻ transport. Large unilamellar vesicles (LUVs) of POPC (mean diameter = 200 nm) were loaded with HPTS (1 mM) and sodium gluconate (100 mM) buffered at pH 7.0 with 10 mM HEPES. The vesicles were suspended in an external solution of sodium gluconate (100 mM) buffered at pH 7.0 with 10 mM HEPES. At the beginning of the experiment, a base pulse of TBAOH (5 mM) was added to create a pH gradient, and the dissipation of the pH gradient induced by the test ionophore (added as a DMSO solution) was monitored by HPTS fluorescence. Lipid concentration for fluorescence measurement was 0.10 mM. Dose-dependent Hill plot analysis was performed to obtain a Hill coefficient (*n*) and an effective concentration to reach 50% of maximum transport (EC₅₀) at 200 s for each ionophore. Ionophore concentrations are shown as ionophore-to-lipid molar ratios. See Figures S23–S29 for Hill plots.

^cA fluorescence assay for H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport. The internal and external medium used was NMDG-Cl (100 mM) buffered at pH 7.0 with 10 mM HEPES, and the base pulse used was NMDG (5 mM). The other conditions are the same as in the TBAOH assay. See Figures S34–S55 for Hill plots. ^dIn DMSO, reported by Jakab et al.⁴¹

^eNot determined.

 f In acetonitrile-water (9:1 v/v with 0.1 M TBAPF₆), determined by potentiometric titrations.

⁹No transport at 1 mol % concentration. Higher concentrations were not tested because of interference with HPTS fluorescence. See Figure S30.

^hIn water, reported by Šturdík et al.⁴³

ⁱNo transport even at 100 mol % concentration. See Figure S56.

Several simple ureas/thioureas **1–6**, prodigiosin, and a known protonophore carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP; Figure 1) were tested, and the results are presented in Table 1.

Although prodigiosin was extremely active in the NMDG-Cl assay, corresponding to H^+/Cl^- symport or Cl^-/OH^- antiport, it could not dissipate the pH gradient in the TBAOH assay (Table 1), consistent with its inability to facilitate electrogenic transport processes.¹⁷ This conclusion is consistent with the lack of protonophoric activity or alteration of cellular ATP levels reported for prodigiosins.^{14,15} A likely interpretation of these data is that the charged, protonated form of prodigiosin is unable to move through the membrane in the absence of a transportable anion (Figure 2A, right). Consistent with this idea, prodigiosin failed to facilitate electrogenic Cl⁻ transport (see the section Coupling between Cationophores and Anionophores: Direct Evidence of "Valinomycin-likeness"). CCCP transports H⁺, but not Cl⁻, and therefore was active in the TBAOH assay and silent in the NMDG-Cl assay (Table 1). By contrast, the neutral hydrogen-bond donors **1–6** responded in both assays (Table 1). Transport rates were essentially unaffected by switching the external buffer from HEPES to phosphate (Figure S33), indicating that transport of the buffer is unlikely. Further evidence for H⁺/OH⁻ transport that completely rules out buffer

transport is afforded by the observation of H^+/OH^- transport under buffer-free conditions (Supplemental Information, Section 11.1). Thus, like the protonophore CCCP, ureas and thioureas **1–6** facilitated electrogenic H^+/OH^- transport.

The molecular mechanism for H⁺ conductance induced by weak acid protonophores such as CCCP involves the transmembrane movement of both neutral and deprotonated anionic forms of the protonophore (Figure 2C, left).³⁹ Anionophores acting as hydrogen-bond donors might adopt a CCCP-like deprotonation mechanism. This process, coupled with electrogenic Cl⁻ transport, would lead to overall H⁺/Cl⁻ symport in the NMDG-Cl assay. Alternatively, anionophores might produce the same effect as protonophores by reversibly binding and releasing OH⁻ or hydrated OH⁻ (Figure 2C, right). To identify the mechanism responsible for the protonophoric activity, ureas/thioureas and their mono-N-methylated analogs were compared in both assays. N-Methylation is expected to dramatically weaken anion (Cl⁻ or OH⁻) binding and transport because only a single NH hydrogen-bond donor is present.⁴⁰

In the NMDG-Cl assay, N-methylation dramatically decreased the activities of both urea 1 and thiourea 4, consistent with weakened Cl⁻ binding by N-methylation. However, results from the TBAOH assay were more complex; urea 1 was still far more active than N-methyl urea 2, but thiourea 4 and its N-methyl analog 5 showed similar activities (Table 1). To rationalize these effects, pK_a values of some compounds were determined by potentiometric titrations in acetonitrile-water (9:1 v/v), and literature pK_a values⁴¹ (in DMSO) were tabulated (Table 1). Electrostatic potential values at the nitrogen nucleus (EPN)⁴² obtained with density functional theory calculations (see Supplemental Information, Section 7.1) were used to compare acidity when the pK_a values were too high for determination by potentiometric titration.

Because of its high acidity, a significant proportion of compound 4 was deprotonated under the experimental conditions (external pH \sim 8 after base pulse), likely allowing H⁺ transport in the same manner as CCCP.⁴³ Despite its weaker acidity, compound 5 also existed in equilibrium with its deprotonated form in water, and the following lines of evidence lead us to suggest that 5 transported H⁺ via a CCCP-like mechanism: (1) 5 was much better at transporting H⁺/OH⁻ than Cl⁻ (Table 1, compare EC_{50} values in the two assays), and (2) 5 showed a Hill coefficient of \sim 2 in the NMDG-Cl assay, suggesting Cl⁻ transport via a 2:1 (5·Cl⁻) complex. However, the different Hill coefficient of \sim 1 in the TBAOH assay suggests that an alternative process from anion binding was occurring. We attribute this process to a CCCP-like deprotonation mechanism. These observations for 5 are in sharp contrast to other ureas/thioureas, which show EC₅₀ values of the same order of magnitude and similar Hill coefficients in the two assays (Table 1). When comparing 4 and 5, the disadvantage of the weaker acidity of 5 is likely compensated by its higher lipophilicity (it has been shown that carrier lipophilicity is favorable for membrane transport²⁵) and weaker binding to the lipid phosphate head group, leading to similar activities of 4 and 5 in H $^+$ transport. It is also possible that 4 transports OH $^-$ as a result of stronger anion binding, different from the H⁺ transport mechanism of 5.

Ureas 1–3 have pK_a values higher than 13 (Table 1; although we were unable to determine the pK_a of 2 by potentiometric titration, 2 is less acidic than 1, as indicated by their EPN values). Therefore, H⁺ transport by a deprotonation mechanism at physiological pH is less likely for ureas 1–3 than thioureas 4 and 5. The higher activity of 1 than of the *N*-methyl urea 2 in the TBAOH assay seems to support the hypothesis that 2 and possibly also 1 facilitated OH⁻ transport by hydrogen bonding to OH⁻.

However, because N-methylation weakens acidity, comparison between 2 and 3 is more conclusive. When compared with 3, compound 2 is more acidic on the basis of their EPN values (Table 1), and it is also more lipophilic. Therefore, if both 2 and 3 facilitated H^+ transport via deprotonation, then 2 would be more active than 3 in the TBAOH assay. However, the TBAOH assay revealed that 2 had a lower activity than 3, highlighting the importance of two NH hydrogen-bond donors, which supports the hypothesis of OH⁻ transport. This is also supported by the similarly high Hill coefficients in the two assays, in the cases of 2, 3, and 6, indicating the complexation of both Cl⁻ and OH⁻ by more than one receptor molecule. Although 3 and 6 contain two NH hydrogen-bond donors, they showed high Hill coefficients, presumably because of the less acidic nature of the NH hydrogen-bond donors (less electron-deficient anion binding site) than of those in 1 and 4, and therefore they needed more carrier molecules to transport Cl⁻ and OH⁻. This is supported by the report that relatively weak halogen-bonding anionophores show high Hill coefficients in anion transport.¹⁸ The above-mentioned results demonstrate that OH⁻ (or hydrated OH⁻) transport is the more likely pathway for less acidic anionophores to facilitate H⁺/OH⁻ transport, while the more acidic 5 can deprotonate in water and thereby transport H^+ in the same way as CCCP.

It should be noted that the OH⁻ complex (1:1) and the hydrated deprotonated form of a urea/thiourea are structurally similar, differing only by small changes in the position of the proton (Figure 2D). This difference would disappear in the case of a single-well [N···H···OH]⁻ hydrogen bond (similar to the [F···H···F]⁻ ion).⁴⁴ This highlights the fact that, when direct deprotonation of a receptor cannot take place, OH⁻ can nonetheless form a strong complex with a less acidic receptor. This might help to explain the surprising observation that synthetic anionophores may transport OH⁻ as efficiently as Cl⁻, despite the hydrophilicity of OH⁻. We next show, however, that by rational design of the anionophore structure, we can reduce OH⁻ or H⁺ transport in relation to Cl⁻ transport.

Cl⁻ > H⁺/OH⁻ Selectivity

We investigated whether it is possible for anionophores to possess selectivity for electrogenic Cl⁻ transport over H⁺/OH⁻ (including electrogenic and non-electrogenic pathways) transport, which we refer to as $CI^- > H^+/OH^-$ selectivity. Because of its perfect $K^+ > H^+/OH^-$ selectivity, low concentrations of valinomycin do not facilitate K^+/H^+ antiport unless a protonophore is present.⁴⁵ On the basis of this rationale, an HPTS assay for $Cl^- > H^+/OH^-$ selectivity was devised and employed (Figure 3B). In this modified NMDG-Cl assay, we studied the effects of the proton channel gramicidin D on the rate of pH-gradient dissipation (indicating H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport) induced by anionophores. If H⁺/OH⁻ transport was rate limiting (i.e., electrogenic Cl^{-} transport was faster than H^{+}/OH^{-} transport, namely $Cl^- > H^+/OH^-$ selectivity), pH-gradient dissipation would be significantly accelerated by gramidicin, which facilities electrogenic H⁺ transport. Conversely, if Cl⁻ transport was rate limiting (or if the anionophore did not facilitate electrogenic Cl⁻ transport faster than H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport), the rate of pHgradient dissipation would be unaffected by gramidicin. The neutral proton channel gramicidin was employed to prevent potential intermolecular interaction between anionophores and proton carriers such as carbonyl cyanide phenylhydrazones. The use of inert NMDG⁺ (Figure 1) as the cation component precluded gramicidin itself from dissipating the pH gradient by facilitating M⁺/H⁺ antiport. The ratio between EC₅₀ values obtained in the absence and presence of gramicidin (selectivity [S] value shown in Table 2) was used to quantify $Cl^- > H^+/OH^-$ selectivity. It is important to note that the selectivity value obtained is a relative value dependent on the

Anionophore	Without Gra		With Gra (0.1 mol %)		Selectivity (<i>S</i>) ^b
	n	EC ₅₀ (mol %)	n	EC ₅₀ (mol %) ^a	
1	1.4 ^c	0.046 ^c	1.4	0.043	1.1
2	2.4 ^c	0.61 ^c	1.9	0.29	2.1
3	2.3°	0.16 ^c	2.0	0.11	1.4
4	1.2 ^c	0.015 ^c	1.2	0.015	1.0
5	1.9°	0.26 ^c	1.8	0.33	0.8
6	3.2°	20 ^c	3.1	13	1.5
7	2.5	7.9	2.7	5.9	1.3
8	1.3	0.00074	1.3	0.00085	0.9
9	1.3	0.042	1.1	0.0030	14
10	1.2	0.0011	1.2	0.0012	0.9
11	1.3	0.11	1.3	0.0028	39
12	1.2	0.067	1.4	0.00086	78
13	-	too inactive	-	too inactive	_d
14	1.2	9.5	1.0	1.3	7.4
15	1.5	0.25	1.2	0.077	3.3
16	1.3	0.016	1.4	0.017	0.9
17	-	too inactive	0.9	0.089	_e
18	1.4	0.18	1.2	0.0017	100
Trihexylamine	1.1	0.026	1.1	0.023	1.1
I ₂	1.6	6.9	1.5	6.1	1.1
Prodigiosin	1.4 ^c	0.000061 ^c	1.3	0.000059	1.0
Mn(TPP)Cl	1.1	0.0051	1.2	0.0045	1.1

Table 2. Summary of Cl⁻ Transport Activity and Cl⁻ > H⁺/OH⁻ Selectivity

Hill plot analysis of anionophores **1–18**, trihexylamine (Hex₃N), iodine (I₂), prodigiosin (Prod), and Mn(TPP) CI in the NMDG-CI assay in the absence and presence of the proton channel gramicidin D (Gra). The NMDG-CI assay is a fluorescence assay for H^+/CI^- symport or CI⁻/OH⁻ antiport. Large unilamellar vesicles (LUVs) of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; mean diameter, 200 nm) were loaded with HPTS (1 mM) and NMDG-CI (100 mM), and buffered at pH 7.0 with 10 mM HEPES. The vesicles were suspended in an external solution of NMDG-CI (100 mM) buffered at pH 7.0 with 10 mM HEPES. At the beginning of the experiment, a base pulse of NMDG (5 mM) was added to create a pH gradient, and the dissipation of the pH gradient induced by the test ionophores (added as a DMSO solution, or ethanol and acetonitrile solutions in some cases) was monitored by HPTS fluorescence. Lipid concentration for fluorescence measurement was 0.10 mM. Dose-dependent Hill plot analysis was performed to obtain a Hill coefficient (*n*) and an effective concentration to reach 50% of maximum transport (EC₅₀) at 200 s for each ionophore. Ionophore concentrations are shown as ionophore-to-lipid molar ratios. See Figures S34–S55 for Hill plots.

 ${}^{a}EC_{50}$ in the presence of Gra indicates Cl⁻ transport activity, since without Gra, H⁺/OH⁻ transport may be rate limiting. Note that the "activity" here is the "total activity" of electroneutral H⁺/Cl⁻ symport (or Cl⁻/OH⁻ antiport) and electrogenic Cl⁻ transport. The Gra concentration used (0.1 mol %) has been optimized to ensure maximum acceleration of pH-gradient dissipation for a Cl⁻ > H⁺/OH⁻-selective anionophore.

 ${}^{b}CI^{-} > H^{+}/OH^{-}$ selectivity (S) is quantified by the EC₅₀ in the absence of Gra divided by EC₅₀ in the presence of Gra; an S value of 1 indicates H⁺/OH⁻ transport faster than Cl⁻ transport, i.e., no selectivity. ^cThe same data as shown in Table 1.

^dToo inactive for Hill plot analysis. A single-concentration comparison showed no selectivity (Figure S46). ^eNo S value is given because, without gramicidin, **17** was too inactive for Hill plot analysis. This indicates a very high $CI^- > H^+/OH^-$ selectivity that cannot be quantified by this method.

 CI^- concentration employed. It should not be regarded as the absolute ratio between the permeability values of CI^- and H^+/OH^- . Table 2 presents selectivity data for the 22 anionophores shown in Figure 1 (refer to Section 3 in the

Supplemental Information for synthesis of new compounds), the majority of which are hydrogen-bond donors.

Prodigiosin showed no selectivity, consistent with its reported non-electrogenic nature (Table 2).¹⁷ The commercially available Cl⁻ ionophore Mn(TPP)Cl¹⁹ was also unselective, likely because the Lewis acid metal center facilitated efficient OH⁻ transport. Simple monopodal ureas/thioureas **1–7** and a squaramide **8** showed rather weak to no selectivity.

While testing the $Cl^- > H^+/OH^-$ selectivity of anionophores, we serendipitously identified that the tripodal thiourea 9,46 but not its fluorinated analogs,25 was both relatively highly selective (14-fold) and comparatively active (Table 2). New tripodal thioureas were therefore synthesized in an attempt to improve both activity and selectivity. Interestingly, adding electron-withdrawing cyano substituents to 9 (compound 10) led to loss of selectivity (pK_a of 10 was 10.5 in 9:1 acetonitrile-water; Figure S21). Conversely, changing the phenyl group to an *n*-pentyl group (compound 11) noticeably improved selectivity to 39-fold (Table 2). This result suggests that selectivity is sensitive to the acidity of the NH groups. By changing the linear n-pentyl group to the bulky tert-pentyl group, both activity and selectivity further improved from 11 to 12 (Table 2). Of note, 12 showed a remarkable selectivity of 78-fold, whereas its Cl^- transport activity matched that of squaramide 8,⁴⁷ one of our most active anionophores reported to date. Despite the presence of a basic tertiary amine group and therefore the possibility of Cl⁻ transport as a protonated ion-pair complex for the tripodal thioureas (Figure S63), pH-dependent aniontransport studies (Figure S65) demonstrated that the tripodal thioureas actually transported Cl⁻ in their neutral forms (forming anionic complexes), unlike the "HCl receptors" prodigiosin and trihexylamine. Consistent with this idea, the crystal structure of the 12 tetraethylammonium chloride (TEACI) complex (Figure 4A) demonstrates the ability of the neutral form of 12 to encapsulate Cl⁻ with six NH···Cl⁻ hydrogen bonds (see Figure S22 for a ball-and-stick model).

Two effects arising from the tripodal anionophores might be key to their superior $Cl^- >$ H⁺/OH⁻ selectivity in comparison to that of monopodal anionophores: (1) enhanced affinity for Cl⁻ due to the chelate effect⁴⁸ (e.g., compare the Cl⁻ affinity of 7 [K = 14 M⁻¹]⁴⁹ and 9 [K = 190 M^{-1}]⁴⁶ in DMSO-d₆/0.5% H₂O) and (2) anion encapsulation enforcing a high degree of anion desolvation. Although the Hill coefficient of ~2.6 in the case of 7 (Table 2) suggests that Cl⁻ is likely sequestered by three thiourea molecules, like binding of Cl⁻ within the tripodal thiourea cage 9, the alkyl spacer in 9 enforces a higher degree of Cl⁻ desolvation than does the 3:17 · Cl⁻ complex, where the three thiourea moieties are not interconnected. To rationalize these effects, the selectivity of bisthioureas 13 and 14 was examined. Both compounds can bind Cl^- via four $NH\cdots Cl^-$ hydrogen bonds, but the long alkyl spacer in 14 enforces a higher degree of anion desolvation (compare Figures 4B and 4C). Indeed, appending an additional thiourea moiety led to enhancement of Cl⁻ affinities of both 13 (K_1 = 150 M⁻¹, K_2 = 3.4 M⁻¹; Figure S16) and 14 $(K_1 = 530 \text{ M}^{-1}, K_2 = 6.2 \text{ M}^{-1};$ Figure S18) in DMSO- $d_6/0.5\%$ H₂O in relation to that of 7 ($K = 14 \text{ M}^{-1}$).⁴⁹ But, improvement of selectivity (relative to 7, 1.3-fold selectivity) was only observed for 14 (7-fold selectivity), whereas 13 demonstrated no selectivity (Figure S46), underscoring the importance of anion encapsulation. This idea is also supported by the higher selectivity of 12 than that of 11, given that the bulky alkyl substituents in 12 make Cl⁻ less solvent accessible than linear alkyl substituents in 11. The exact reason behind the benefit of anion encapsulation for selectivity is unclear at the moment. We propose that this could be related to the higher hydration enthalpy of OH⁻ than of Cl^{-,50} When the dehydration is caused by the part of the receptor that does not

CellPress

Chem



Figure 4. Space-Filling Representations of Crystallographic or Optimized Structures of Cl Complexes

(A) X-ray crystal structure of the **12**·TEACI complex (CCDC: 1431251; see Section 8 in the Supplemental Information for details).

(B and C) Molecular models of Cl $^-$ complexes of (B) 14 and (C) 13, optimized using the semi-empirical PM6 method.

Atom colors are as follows: gray, C; white, H; blue, N; yellow, S; and green, Cl.

significantly contribute enthalpically to binding anions, such as a spacer group, the anion-binding dehydration enthalpic cost for receptors that enforce a higher degree of dehydration will be higher than for those enforcing a lower degree of dehydration. This may confer greater selectivity on anionophores that encapsulate anions to a higher degree.

Although, in general, more acidic hydrogen-bond donors are favorable for anion binding and transport (in terms of activity), ^{49,51} Table 2 demonstrates that Cl⁻ > H⁺/OH⁻ selectivity is severely compromised when more acidic hydrogen-bond donors are present. For example, the selectivity sequence of 11 > 9 \gg 10 illustrates this effect. An obvious disadvantage of higher-acidity anionophores is the possibility of H⁺ transport via a CCCP-like deprotonation mechanism (Figure 2C, left). Deprotonation is, however, not the only reason for the observed negative correlation between selectivity and acidity, given that even the halogen-bonding-based anionophore iodine showed no selectivity despite its inability to transport H⁺ by deprotonation of the receptor (Table 2). It seems that more acidic (thus more electron-deficient) anionophores favor the transport of the more charge-dense OH⁻ over the less charge-dense Cl⁻. This idea is consistent with the previous report that a CH hydrogen-bond-based anionophore, which is much less acidic than NH hydrogen-bond receptors, showed a high selectivity for Cl⁻ over the more charge-dense HCO₃⁻.⁵² In contrast, a highly acidic bisurea anionophore lacked the Cl⁻ > HCO₃⁻ selectivity.

The steroid scaffold is known to pre-organize two or three anion-binding motifs, resulting in powerful Cl⁻ ionophores ("cholapods").⁵³⁻⁵⁵ We subjected several

reported and new cholapod-based urea anionophores to the selectivity test. Compound 15⁵³ showed modest selectivity of 3-fold, which was eliminated when a trifluoroacetamide group, ⁵⁴ as an additional hydrogen-bond donor, was introduced (compound 16), likely because of its high acidity (Table 2). However, remarkable selectivity was achieved by 17 and 18, which feature binding-site enclosure (Table 2).⁵⁶ Compound 17 was extremely selective to the extent that H^+/OH^- transport was too inactive for Hill plot analysis. However, compound 17 was not a very active chloride carrier (Table 2). Despite containing an acidic trifluoroacetamide group, the more active 18 demonstrated a surprising 100-fold selectivity (Table 2). Presumably, the advantages of binding-site enclosure, possibly also from a decrease in urea NH acidity due to electron-donating alkoxy substituents, outweigh the disadvantage of an acidic trifluoroacetamide group. It is of interest to note that the Claffinities of 17 (K = $1.2 \times 10^5 \text{ M}^{-1}$) and 18 (K = $3.6 \times 10^5 \text{ M}^{-1}$) were dramatically lower than those of 15 (K = $1.5 \times 10^7 \text{ M}^{-1}$)⁵³ and 16 (K = $2.8 \times 10^8 \text{ M}^{-1}$)⁵⁴ in water-saturated chloroform (for binding-constant determination, see Supplemental Information, Section 5). The higher $CI^- > H^+/OH^-$ selectivity observed with the weakly binding but more encapsulating receptors 17 and 18 than with 15 and 16 again supports our proposition that anion encapsulation to a high degree, but not high-affinity anion binding, benefits selectivity.

Further evidence of the Cl⁻ > H⁺/OH⁻ selectivity of 12 and 18 was demonstrated by their coupling with the proton carrier CCCP to facilitate H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport in HPTS (Figure S62), ion-selective electrode (ISE; Figure S67), and osmoticresponse assays (Figure S70). Thus, 12 and 18 couple with H⁺ transporters irrespective of the H⁺ transport mechanism (channel or carrier). This also suggests that H⁺ transport and Cl⁻ transport are independent processes in this case, ruling out possible intermolecular interactions between a proton transporter and 12 or 18. As a complementary test, an HPTS assay conducted using KCl as the medium demonstrated that the activities of 12 and 18 were unaffected by the presence of the K⁺ ionophore valinomycin (Figure S62), which can replace potentially ratelimiting Cl⁻ transport with faster K⁺ transport as the counterion pathway. This result indicates that Cl⁻ transport is not rate limiting for the pH change⁵⁷ (for further explanation, see Supplemental Information, Section 9.4), which further supports the Cl⁻ > H⁺/OH⁻ selectivity of 12 and 18.

Coupling between Cationophores and Anionophores: Direct Evidence of "Valinomycin-likeness"

Because valinomycin (an electrogenic cationophore) and monensin (a non-electrogenic cationophore) function via fundamentally different mechanisms, we used these compounds to investigate the anion-transport mechanisms of representative anionophores. Ionophore-induced Cl⁻ efflux was measured by ISE from KCl-loaded vesicles suspended in an inert external K₂SO₄ solution. Here, Cl⁻ transport was mainly driven by the large Cl⁻ concentration gradient (300 mM inside and ~0 mM outside), but no measurable Cl⁻ efflux could occur in the presence of an anionophore alone because of the buildup of a membrane potential (if the test anionophore was an electrogenic Cl⁻ carrier) or a pH gradient (if the test anionophore was a H⁺/Cl⁻ symporter or a Cl⁻/OH⁻ antiporter). Valinomycin dissipated the membrane potential from electrogenic CI^- transport⁵⁸ (the K^+ gradient also provided a small additional driving force for CI^- transport), allowing electrically coupled K^+/CI^- flux in the presence of an electrogenic Cl⁻ transporter (Figure 3C).⁵⁹ By contrast, monensin dissipated the pH gradient accumulated by Cl⁻/OH⁻ antiport (or H^+/Cl^- symport) through K^+/H^+ antiport, leading to formal KCl flux in the presence of a H⁺/Cl⁻ symporter or Cl⁻/OH⁻ antiporter (Figure 3D).

CellPress



Figure 5. Coupling between Cationophores and Anionophores to Facilitate Net KCI Flux (A–D) POPC LUVs (mean diameter = 200 nm) were loaded with KCI (300 mM) and K₂SO₄ (200 mM) buffered at pH 7.4 with 5 mM HEPES. The vesicles were suspended in an external solution of K₂SO₄ (200 mM) buffered at pH 7.4 with 5 mM HEPES. Cl⁻ efflux induced by (A) prodigiosin (Prod), (B) 1, (C) 12, or (D) 18 in the absence and presence of valinomycin (Vln, 0.1 mol % with respect to lipid) or monensin (Mon, 0.1 mol %) was monitored using a Cl⁻-selective electrode. All ionophores were added to the vesicle suspensions as DMSO solutions. Detergent was added at 5 min to release all Cl⁻ and calibrate Cl⁻ efflux to 100%. Lipid concentration was 1.0 mM. Ionophore concentrations are shown as ionophore-to-lipid molar ratios. Error bars represent standard deviations from two repeats. The same DMSO, Vln, and Mon controls were used in all figures. See Figures 3C and 3D for schematic illustrations.

Figure 5A shows that prodigiosin coupled with monensin, but not valinomycin, demonstrating that prodigiosin cannot facilitate electrogenic Cl⁻ transport and is thus the anionophore equivalent of the non-electrogenic cationophore monensin. The unselective anionophore 1 coupled with both valinomycin and monensin (Figure 5B), demonstrating that 1 can facilitate both electrogenic Cl⁻ transport and electroneutral Cl⁻/OH⁻ antiport (or H⁺/Cl⁻ symport) at the same concentration. The same transport rates observed in the presence of valinomycin and monensin suggest that Cl⁻ transport is the same rate-limiting process, also ruling out potential

transport acceleration via ion pairing between the valinomycin·K⁺ complex and the 1·Cl⁻ complex. Taking into account that 1 also facilitated electrogenic H⁺/OH⁻ transport (Table 1), 1 can be regarded as a charge-inversed equivalent of "valinomycin + CCCP" (but with a much lower activity). Importantly, the selective anionophores 12 and 18 coupled with valinomycin, but not monensin (Figures 5C and 5D), indicating that they can facilitate electrogenic Cl⁻ transport with little H⁺/OH⁻ transport at the same concentration. Potential transport acceleration via ion pairing in the case of 12 and 18 coupling with valinomycin is unlikely given that valinomycin-likeness" of selective anionophores 12 and 18 is thereby firmly established. Thus, the results reveal that prodigiosin, 1, and 12 (18) are representative examples of three types of anionophores with different functions (for further details, see Supplemental Information, Sections 9.4, 12, and 14).

Anion-Transport and pH-Perturbation Studies in Cells

To begin to investigate the action of anionophores in cells, we used model systems to study the effects of anionophores on anion transport, pH-gradient disruption, and cytotoxicity. For these experiments, we used Fischer rat thyroid (FRT) cells, a cell line used to investigate epithelial ion transport, ⁶⁰ and A549 cells, a cell line employed for pH-gradient disruption and cytotoxicity studies.²⁶ To measure anionophore-mediated Cl⁻ transport in FRT cells, we used FRT cells engineered to express the halide-sensitive yellow-fluorescent protein, YFP-H148Q/I152L.^{11,61,62} Facilitated Cl⁻ transport through the plasma membrane was measured indirectly by I⁻ entry into the cells coupled with the exit of intracellular Cl⁻, leading to quenching of YFP fluorescence by I⁻.¹¹ Compound **18** was inactive in this assay (data not shown), likely because of low deliverability.¹¹ By contrast, Figure 6A demonstrates that compound **12** mediated anion transport with high activity (similar to the active anionophores reported previously).¹¹

We performed cytotoxicity studies in human lung adenocarcinoma (A549) cells. Compound **12** exhibited modest toxicity to A549 cells with a half-maximum inhibitory concentration (IC₅₀, determined after treatment for 24 hr) of 43 \pm 4 μ M. Interestingly, analog **10** was significantly less toxic with IC₅₀ > 100 μ M (Supplemental Information, Section 13). Possibly related to this result, we found that **10** was unable to facilitate electrogenic Cl⁻ transport in LUV assays (Figure S73A).

In lysosomes, an acidic luminal pH is established by H⁺ pumping into lysosomes by V-ATPase and a counterion pathway, which is likely Cl⁻/H⁺ antiport via ClC-7.^{63,64} Prodigiosin is known to neutralize lysosome pH by electroneutral H⁺/Cl⁻ symport out of lysosomes, which is a possible cause of its toxicity.^{14,15} Compound 12 was tested for its ability to neutralize lysosome pH in A549 cells using the pH-sensitive fluorescent dye acridine orange (AO) to stain lysosomes and endosomes. After treating A549 cells with 50 μ M 12, a concentration close to its IC₅₀, only a small proportion of the orange fluorescence inside the cells disappeared compared with the DMSO control (Figure 6B), suggesting only slight neutralization of lysosomal pH. This result contrasts with recently reported tambjamine-derived anionophores that led to complete disappearance of AO fluorescence in the same cell line at concentrations close to their IC₅₀ (\sim 10 μ M).²⁶ Similarly, analogs of 9 with electronwithdrawing fluorine or trifluoromethyl substituents,²⁵ other anionophores with highly acidic hydrogen-bond donors, ^{23,24} and other tambjamine-derived "HCl receptors"²⁷ exhibited potent lysosomal pH neutralization, although they were tested in different cell lines. Anionophores bearing highly acidic hydrogen-bond donors

в

CellPress





12 (50 µM)





Figure 6. Compound 12 Mediates Anion Transport without Effectively Neutralizing Lysosomal pH in Cells

(A) (Left) Anion transport by FRT cells expressing the halide sensor YFP-H148Q/I152L at the indicated concentrations of compound **12** was determined from the fit of first-order exponential functions to the fluorescence decay elicited by Nal (100 mM). Fluorescence quenching by the anionophore vehicle (1–10 μ M, 0.1% v/v DMSO; 50 μ M, 0.5% v/v DMSO) was subtracted from each test measurement to determine the transport activity of compound **12**. Data are shown as means \pm SEM (n = 35–40 from at least three independent experiments). (Right) Representative time courses of cell fluorescence. Values of cell fluorescence were normalized to the fluorescence intensity at *t* = 0 s.

(B) Acridine orange staining of human lung adenocarcinoma (A549) cells treated with DMSO control (0.5% v/v) or compound **12** (50 μ M, added in DMSO). The acridine orange staining was performed four times in duplicate, and similar results were obtained.

(e.g., 8 and 10) or functioning as "HCI receptors" (e.g., prodigiosin and trihexylamine) are unfavorable for selectivity, as demonstrated in our examples. The results reported here are consistent with the Cl⁻ > H⁺/OH⁻ selectivity of 12, indicative of low activity in facilitating H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport, processes required for lysosomal pH neutralization. The selectivity, however, is not as perfect as the K⁺ > H⁺/OH⁻ selectivity of valinomycin (Figure S61A); this possibly explains the minor lysosomal pH neutralization observed.

The change in intracellular (cytosolic) pH (pH_i) of A549 cells was also measured using the intracellular pH indicator SNARF-1 after anionophore treatment. Compound **12** induced a decrease in pH_i of 0.42 \pm 0.04 pH units in this cancerous cell line. The exact cause of the decrease in pH_i is unclear at this moment. One possibility is Cl⁻/HCO₃⁻ antiport⁴⁶ facilitated by **12**. The decrease in pH_i might also be an indirect consequence of electrogenic Cl⁻ transport facilitated by **12**, which could trigger or couple with other cellular ion transporters involved in pH_i regulation under the experimental conditions.

Conclusions

The application of synthetic anionophores to biological systems is still at an early stage. However, in this article, we report a significant step forward by first developing assays to measure the selectivity of anionophores for CI^- over H^+/OH^- and second developing electrogenic anionophores with high selectivity. Specifically, we have demonstrated that many small-molecule anionophores are capable of facilitating electrogenic H^+/OH^- transport. By comparing a series of ureas/thioureas and mono-N-methylated ureas/thioureas, we provide evidence that receptors acting through hydrogen bonding can transport H⁺ by a CCCP-like deprotonation mechanism or OH⁻ by hydrogen bonding; the latter pathway is more likely for less acidic hydrogen-bond donors. To emulate the highly selective electrogenic K⁺ carrier valinomycin, we developed the synthetic small-molecule electrogenic Cl⁻ carriers 12 and 18, for which H^+/OH^- transport is suppressed. The remarkable $CI^- > H^+/OH^-$ selectivity of these systems is confirmed by (1) coupling with both proton channel gramicidin and proton carrier CCCP to facilitate H⁺/Cl⁻ symport (or Cl⁻/OH⁻ antiport), as shown by fluorescence, ISE, and osmotic response assays; and (2) coupling with valinomycin, but not with monensin, to facilitate net KCl flux. Our results provide guidelines to identify different anion-transport mechanisms (e.g., electroneutral, electrogenic, and pH/proton-gradient dissipation) and develop anionophores with desired functions by rational design. For example, the ability to encapsulate Cland the absence of highly acidic hydrogen-bond donors seem to be crucial characteristics of electrogenic $Cl^- > H^+/OH^-$ anionophores. As one of the first proven examples of "valinomycin-like" anionophores, 12 was found to facilitate Cltransport in cells without effectively neutralizing lysosome pH, unlike other anionophores that achieve high anion-transport activity by using highly acidic hydrogenbond donors or functioning as prodigiosin-like "HCl receptors." We propose that compounds of this type can play an important role in biomedical research and are potentially more suitable for treating "channelopathies," such as cystic fibrosis, than other unselective anionophores.

EXPERIMENTAL PROCEDURES

HPTS assays based on pH-gradient dissipation¹⁸ were conducted using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) LUVs (mean diameter = 200 nm) loaded with the pH-sensitive fluorescence dye HPTS (1 mM), with the unencapsulated HPTS removed by size-exclusion chromatography. The internal and external solutions used were identical salt solutions (Na⁺-gluconate, NMDG-Cl, (NMDG)₂SO₄, K⁺-gluconate, or KCl, depending on the assay) buffered with 10 mM HEPES (pH 7.0). For each measurement, a 2 mL sample containing 0.1 mM lipid was used. To this vesicle suspension was added a base pulse (TBAOH, NaOH, KOH, or NMDG, depending on the assay; final base concentration = 5 mM) to generate a transmembrane pH gradient. At the beginning of each measurement, a DMSO solution (in some cases ethanol and acetonitrile solutions were used) of the test ionophore was added, and the ratiometric fluorescence of

HPTS ($\lambda_{ex} = 460 \text{ nm}$, $\lambda_{em} = 510 \text{ nm}$, base form divided by $\lambda_{ex} = 403 \text{ nm}$, $\lambda_{em} = 510 \text{ nm}$, acid form) was recorded. In cases where an assisting ionophore (e.g., gramicidin D) was used, the assisting ionophore was added to the vesicle suspension after the addition of the base pulse and prior to the addition of the test ionophore. At 200 s, a detergent was added to destroy the pH gradient and calibrate fluorescence. The fluorescence ratio was normalized to a fractional value. Dose-dependent Hill plot analyses were performed to obtain Hill coefficients (*n*) and EC₅₀ (200 s) values.

ISE assays were conducted using POPC LUVs (mean diameter = 200 nm) loaded with NH₄Cl, KCl, or NaCl (300 mM for salt-efflux experiments and 500 mM for anion-exchange experiments) and suspended in a Cl⁻-free external solution containing Na₂SO₄, K₂SO₄, or NaNO₃ and a buffer. The unencapsulated Cl⁻ was removed by dialysis. For each measurement, a 5 mL sample containing 1 mM lipid was used. At the beginning of each measurement, a DMSO solution (or ethanol solution for trihexylamine) of the test anionophore was added to the vesicle suspension, and Cl⁻ efflux was monitored by a Cl⁻-selective electrode. In cases where an assisting ionophore (CCCP, valinomycin, or monensin) was used, the assisting ionophore. At 5 min, a detergent was added to lyse the vesicles and release all Cl⁻ to calibrate Cl⁻ efflux to 100%.

For further information about the different HPTS and ISE assays employed, see Sections 9 and 10 in the Supplemental Information.

For details of the biological studies, including anionophore-mediated anion transport, cytotoxicity, and intracellular pH measurements, see Section 13 in the Supplemental Information.

ACCESSION NUMBERS

The accession number for the **12**·TEACl complex reported in this article is CCDC: 1431251.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedure, 76 figures, five tables, and a crystallographic data file and can be found with this article online at http://dx.doi.org/10.1016/j.chempr.2016.04.002.

AUTHOR CONTRIBUTIONS

Conceptualization, X.W. and P.A.G.; Methodology, X.W., P.A.G., A.P.D., V.S.-C., R.P.-T., H.L., and D.N.S.; Investigation, X.W., L.W.J., E.N.W.H., A.M.W., V.S.-C., H.L., N.B., and H.V.; Writing – Original Draft, X.W., L.W.J., P.A.G., E.N.W.H., and D.N.S.; Writing – Review & Editing, X.W., P.A.G., E.N.W.H., D.N.S., H.L., A.P.D., H.V., and V.S.-C.; Funding Acquisition, P.A.G., A.P.D., R.P.-T., D.N.S., and Y.-B.J.; Supervision, P.A.G., A.P.D., R.P.-T., D.N.S., and Y.-B.J.

ACKNOWLEDGMENTS

We thank Alan S. Verkman for the generous gift of FRT cells expressing YFP-H148Q/ I152L and Christopher Dias for preparing samples for the FRT assay. This work was supported by EPSRC grants EP/J009687/1, EP/F03623X/1, and EP/J00961X/1, a grant from the Spanish government and the EU (FIS PI13/00089), and a grant from La Marató de TV3 Foundation (20132730). P.A.G. thanks the Royal Society and

the Wolfson Foundation for a Royal Society Wolfson Research Merit Award. X.W. was supported by studentships from the University of Southampton and the China Scholarship Council.

Received: January 15, 2016 Revised: February 3, 2016 Accepted: March 23, 2016 Published: June 9, 2016

REFERENCES AND NOTES

- Matile, S., Vargas Jentzsch, A., Montenegro, J., and Fin, A. (2011). Recent synthetic transport systems. Chem. Soc. Rev. 40, 2453– 2474.
- McNally, B.A., Leevy, W.M., and Smith, B.D. (2007). Recent advances in synthetic membrane transporters. Supramol. Chem. 19, 29–37.
- Pressman, B.C. (1976). Biological applications of ionophores. Annu. Rev. Biochem. 45, 501–530.
- Läuger, P. (1972). Carrier-mediated ion transport: electrical relaxation experiments give insight into the kinetics of ion transport through artificial lipid membrane. Science 178, 24–30.
- Läuger, P. (1985). Mechanisms of biological ion transport — carriers, channels, and pumps in artificial lipid membranes. Angew. Chem. Int. Ed. Engl. 24, 905–923.
- Mollenhauer, H.H., Morré, D.J., and Rowe, L.D. (1990). Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochim. Biophys. Acta Rev. Biomembr. 1031, 225–246.
- Davis, J.T., Okunola, O., and Quesada, R. (2010). Recent advances in the transmembrane transport of anions. Chem. Soc. Rev. 39, 3843– 3862.
- 8. Davis, A.P., Sheppard, D.N., and Smith, B.D. (2007). Development of synthetic membrane transporters for anions. Chem. Soc. Rev. *36*, 348–357.
- 9. Berezin, S.K. (2015). Valinomycin as a classical anionophore: mechanism and ion selectivity. J. Membr. Biol. 248, 713–726.
- Denda, M., Fuziwara, S., and Inoue, K. (2003). Influx of calcium and chloride ions into epidermal keratinocytes regulates exocytosis of epidermal lamellar bodies and skin permeability barrier homeostasis. J. Investig. Dermatol. 121, 362–367.
- Li, H., Valkenier, H., Judd, L.W., Brotherhood, P.R., Hussain, S., Cooper, J.A., Jurček, O., Sparkes, H.A., Sheppard, D.N., and Davis, A.P. (2016). Efficient, non-toxic anion transport by synthetic carriers in cells and epithelia. Nat. Chem. 8, 24–32.
- Shen, B., Li, X., Wang, F., Yao, X., and Yang, D. (2012). A synthetic chloride channel restores chloride conductance in human cystic fibrosis epithelial cells. PLoS One 7, e34694.
- Ko, S.-K., Kim, S.K., Share, A., Lynch, V.M., Park, J., Namkung, W., Van Rossom, W., Busschaert,

N., Gale, P.A., Sessler, J.L., et al. (2014). Synthetic ion transporters can induce apoptosis by facilitating chloride anion transport into cells. Nat. Chem. 6, 885–892.

- Ohkuma, S., Sato, T., Okamoto, M., Matsuya, H., Arai, K., Kataoka, T., Nagai, K., and Wasserman, H.H. (1998). Prodigiosins uncouple lysosomal vacuolar-type ATPase through promotion of H⁺/Cl⁻ symport. Biochem. J. 334, 731–741.
- Kataoka, T., Muroi, M., Ohkuma, S., Waritani, T., Magae, J., Takatsuki, A., Kondo, S., Yamasaki, M., and Nagai, K. (1995). Prodigiosin 25-C uncouples vacuolar type H⁺-ATPase, inhibits vacuolar acidification and affects glycoprotein processing. FEBS Lett. 359, 53–59.
- Seganish, J.L., and Davis, J.T. (2005). Prodigiosin is a chloride carrier that can function as an anion exchanger. Chem. Commun. 5781–5783.
- Sato, T., Konno, H., Tanaka, Y., Kataoka, T., Nagai, K., Wasserman, H.H., and Ohkuma, S. (1998). Prodigiosins as a new group of H⁺/Cl[−] symporters that uncouple proton translocators. J. Biol. Chem. 273, 21455–21462.
- Vargas Jentzsch, A., Emery, D., Mareda, J., Nayak, S.K., Metrangolo, P., Resnati, G., Sakai, N., and Matile, S. (2012). Transmembrane anion transport mediated by halogen-bond donors. Nat. Commun. 3, 905.
- El-Etri, M., and Cuppoletti, J. (1996). Metalloporphyrin chloride ionophores: induction of increased anion permeability in lung epithelial cells. Am. J. Physiol. 270, L386– L392.
- Demaurex, N. (2002). pH homeostasis of cellular organelles. Physiology 17, 1–5.
- Henderson, P.J.F., McGivan, J.D., and Chappell, J.B. (1969). The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes. The role of induced proton permeability. Biochem. J. 111, 521–535.
- Berezin, S.K. (2013). Theoretical modelling of anion transport in liposomes: electrogenic anion exchange as a new paradigm in supramolecular chemistry. Supramol. Chem. 25, 323–334.
- Moore, S.J., Haynes, C.J.E., González, J., Sutton, J.L., Brooks, S.J., Light, M.E., Herniman, J., Langley, G.J., Soto-Cerrato, V., Pérez-Tomás, R., et al. (2013). Chloride, carboxylate and carbonate transport by orthophenylenediamine-based bisureas. Chem. Sci. 4, 103–117.

- Moore, S.J., Wenzel, M., Light, M.E., Morley, R., Bradberry, S.J., Gómez-Iglesias, P., Soto-Cerrato, V., Pérez-Tomás, R., and Gale, P.A. (2012). Towards "drug-like" indole-based transmembrane anion transporters. Chem. Sci. 3, 2501–2509.
- 25. Busschaert, N., Wenzel, M., Light, M.E., Iglesias-Hernández, P., Pérez-Tomás, R., and Gale, P.A. (2011). Structure-activity relationships in tripodal transmembrane anion transporters: the effect of fluorination. J. Am. Chem. Soc. 133, 14136–14148.
- 26. Soto-Cerrato, V., Manuel-Manresa, P., Hernando, E., Calabuig-Fariñas, S., Martínez-Romero, A., Fernández-Dueñas, V., Sahlholm, K., Knöpfel, T., García-Valverde, M., Rodilla, A.M., et al. (2015). Facilitated anion transport induces hyperpolarization of the cell membrane that triggers differentiation and cell death in cancer stem cells. J. Am. Chem. Soc. 137, 15892–15898.
- Hernandez, P.I., Moreno, D., Javier, A.A., Torroba, T., Pérez-Tomás, R., and Quesada, R. (2012). Tambjamine alkaloids and related synthetic analogs: efficient transmembrane anion transporters. Chem. Commun. 48, 1556– 1558.
- Heytler, P.G., and Prichard, W.W. (1962). A new class of uncoupling agents — Carbonyl cyanide phenylhydrazones. Biochem. Biophys. Res. Commun. 7, 272–275.
- Mitchell, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. 41, 445–502.
- Accardi, A., and Miller, C. (2004). Secondary active transport mediated by a prokaryotic homologue of CIC CI⁻ channels. Nature 427, 803–807.
- **31.** Ashcroft, F.M. (2000). Ion channels and disease: channelopathies (Academic Press).
- Gutknecht, J. (1987). Proton/hydroxide conductance and permeability through phospholipid bilayer membranes. Proc. Natl. Acad. Sci. USA 84, 6443–6446.
- Marcus, Y. (1991). Thermodynamics of solvation of ions. Part 5.-Gibbs free energy of hydration at 298.15 K. J. Chem. Soc. Faraday Trans. 87, 2995–2999.
- Busschaert, N., Elmes, R.B.P., Czech, D.D., Wu, X., Kirby, I.L., Peck, E.M., Hendzel, K.D., Shaw, S.K., Chan, B., Smith, B.D., et al. (2014). Thiosquaramides: pH switchable anion transporters. Chem. Sci. 5, 3617–3626.

- Matile, S., and Sakai, N. (2012). The characterization of synthetic ion channels and pores. In Analytical Methods in Supramolecular Chemistry, 2 ed., C.A. Schalley, ed. (Weinheim: Wiley-VCH), pp. 711–742.
- Berezin, S.K. (2014). Synthetic anionophores for basic anions as "presumably, OH⁻/Cl⁻ antiporters": from the synthetic ion channels to multi-ion hopping, anti-Hofmeister selectivity, and strong positive AMFE. J. Membr. Biol. 247, 651–665.
- Bakeeva, L.E., Grinius, L.L., Jasaitis, A.A., Kuliene, V.V., Levitsky, D.O., Liberman, E.A., Severina, I.I., and Skulachev, V.P. (1970). Conversion of biomembrane-produced energy into electric form. II. Intact mitochondria. Biochim. Biophys. Acta 216, 13–21.
- Bhosale, S., and Matile, S. (2006). A simple method to identify supramolecules in action: Hill coefficients for exergonic self-assembly. Chirality 18, 849–856.
- McLaughlin, S.G., and Dilger, J.P. (1980). Transport of protons across membranes by weak acids. Physiol. Rev. 60, 825–863.
- Andrews, N.J., Haynes, C.J.E., Light, M.E., Moore, S.J., Tong, C.C., Davis, J.T., Harrell, W.A., Jr., and Gale, P.A. (2011). Structurally simple lipid bilayer transport agents for chloride and bicarbonate. Chem. Sci. 2, 256–260.
- Jakab, G., Tancon, C., Zhang, Z., Lippert, K.M., and Schreiner, P.R. (2012). (Thio)urea organocatalyst equilibrium acidities in DMSO. Org. Lett. 14, 1724–1727.
- Liu, S., and Pedersen, L.G. (2009). Estimation of molecular acidity via electrostatic potential at the nucleus and valence natural atomic orbitals. J. Phys. Chem. A 113, 3648–3655.
- 43. Šturdík, E., Ďurčová, E., Mikeš, V., Dadák, V., Baláž, Š., Sulo, P., and Marián, A. (1987). Acidobasicity, reactivity, lipophilicity, and ability of phenylhydrazonopropanedinitriles to disturb the membrane potential. Collect. Czech. Chem. Commun. 52, 2819–2825.
- Grabowski, S.J. (2011). What is the covalency of hydrogen bonding? Chem. Rev. 111, 2597– 2625.

- Carafoli, E., and Rossi, C.S. (1967). The effect of dinitrophenol on the permeability of the mitochondrial membrane. Biochem. Biophys. Res. Commun. 29, 153–157.
- Busschaert, N., Gale, P.A., Haynes, C.J.E., Light, M.E., Moore, S.J., Tong, C.C., Davis, J.T., and Harrell, W.A., Jr. (2010). Tripodal transmembrane transporters for bicarbonate. Chem. Commun. 46, 6252–6254.
- Busschaert, N., Kirby, I.L., Young, S., Coles, S.J., Horton, P.N., Light, M.E., and Gale, P.A. (2012). Squaramides as potent transmembrane anion transporters. Angew. Chem. Int. Ed. Engl. 51, 4426–4430.
- Hunter, C.A., and Anderson, H.L. (2009). What is cooperativity? Angew. Chem. Int. Ed. Engl. 48, 7488–7499.
- Busschaert, N., Bradberry, S.J., Wenzel, M., Haynes, C.J.E., Hiscock, J.R., Kirby, I.L., Karagiannidis, L.E., Moore, S.J., Wells, N.J., Herniman, J., et al. (2013). Towards predictable transmembrane transport: QSAR analysis of anion binding and transport. Chem. Sci. 4, 3036–3045.
- 50. Smith, D.W. (1977). Ionic hydration enthalpies. J. Chem. Educ. 54, 540–542.
- Gale, P.A., Tong, C.C., Haynes, C.J.E., Adeosun, O., Gross, D.E., Karnas, E., Sedenberg, E.M., Quesada, R., and Sessler, J.L. (2010). Octafluorocalix[4]pyrrole: a chloride/ bicarbonate antiport agent. J. Am. Chem. Soc. 132, 3240–3241.
- 52. Lisbjerg, M., Valkenier, H., Jessen, B.M., Al-Kerdi, H., Davis, A.P., and Pittelkow, M. (2015). Biotin[6]uril esters: chloride-selective transmembrane anion carriers employing C—H…anion interactions. J. Am. Chem. Soc. 137, 4948–4951.
- 53. Koulov, A.V., Lambert, T.N., Shukla, R., Jain, M., Boon, J.M., Smith, B.D., Li, H., Sheppard, D.N., Joos, J.-B., Clare, J.P., et al. (2003). Chloride transport across vesicle and cell membranes by steroid-based receptors. Angew. Chem. Int. Ed. Engl. 42, 4931–4933.
- McNally, B.A., Koulov, A.V., Lambert, T.N., Smith, B.D., Joos, J.-B., Sisson, A.L., Clare, J.P., Sgarlata, V., Judd, L.W., Magro, G., et al. (2008). Structure–activity relationships in cholapod

anion carriers: enhanced transmembrane chloride transport through substituent tuning. Chem. Eur. J. 14, 9599–9606.

- 55. Valkenier, H., Judd, L.W., Li, H., Hussain, S., Sheppard, D.N., and Davis, A.P. (2014). Preorganized bis-thioureas as powerful anion carriers: chloride transport by single molecules in large unilamellar vesicles. J. Am. Chem. Soc. 136, 12507–12512.
- Judd, L.W., and Davis, A.P. (2010). From cholapod to cholaphane transmembrane anion carriers: accelerated transport through binding site enclosure. Chem. Commun. 46, 2227– 2229.
- Weiss, L.A., Sakai, N., Ghebremariam, B., Ni, C., and Matile, S. (1997). Rigid rod-shaped polyols: functional nonpeptide models for transmembrane proton channels. J. Am. Chem. Soc. 119, 12142–12149.
- Baker, J.L., Sudarsan, N., Weinberg, Z., Roth, A., Stockbridge, R.B., and Breaker, R.R. (2012). Widespread genetic switches and toxicity resistance proteins for fluoride. Science 335, 233–235.
- Moore, S.J., Fisher, M.G., Yano, M., Tong, C.C., and Gale, P.A. (2011). A dual host approach to transmembrane transport of salts. Chem. Commun. 47, 689–691.
- Sheppard, D.N., Carson, M.R., Ostedgaard, L.S., Denning, G.M., and Welsh, M.J. (1994). Expression of cystic fibrosis transmembrane conductance regulator in a model epithelium. Am. J. Physiol. 266, L405–L413.
- Verkman, A.S., and Galietta, L.J.V. (2009). Chloride channels as drug targets. Nat. Rev. Drug Discov. 8, 153–171.
- Galietta, L.V.J., Jayaraman, S., and Verkman, A.S. (2001). Cell-based assay for highthroughput quantitative screening of CFTR chloride transport agonists. Am. J. Physiol. Cell Physiol. 281, C1734–C1742.
- Ishida, Y., Nayak, S., Mindell, J.A., and Grabe, M. (2013). A model of lysosomal pH regulation. J. Gen. Physiol. 141, 705–720.
- 64. Graves, A.R., Curran, P.K., Smith, C.L., and Mindell, J.A. (2008). The Cl[−]/H⁺ antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. Nature 453, 788–792.

CellPress